

# Misexpression of the white (*w*) gene triggers male–male courtship in *Drosophila*

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**ABSTRACT** We report here that the general ectopic expression of a tryptophan/guanine transmembrane transporter gene, white (*w*), induces male–male courtship in *Drosophila*. Activation of a *hsp-70*/miniwhite (mini-*w*) transgene in mature males results in a marked change in their sexual behavior such that they begin to vigorously court other mature males. In transformant populations containing equal numbers of both sexes, most males participate, thus forming male–male courtship chains, circles, and lariats. Mutations that ablate the *w* transgene function also abolish this inducible behavior. Female sexual behavior does not appear to be altered by ectopic *w* expression. By contrast, when exposed to an active homosexual courtship environment, non-transformant males alter their behavior and actively participate in the male–male chaining. These findings demonstrate that, in *Drosophila*, both genetic and environmental factors play a role in male sexual behavior.

First reported in 1910 by Morgan, the white (*w*) gene has, over the decades, served as a prototype for numerous studies concerning gene regulation, insertional mutagenesis, and the behavioral analysis of mutants (1–7). Located at the distal end of the X chromosome (8), its 2.6-kb major transcript (9–11) is predicted to encode a 687-amino acid member of the ATP-binding, transmembrane, transporter superfamily (12, 13), which functions in the passage of the ommochrome and drospterin pigment precursors, tryptophan and guanine (respectively) across membranes (14, 15). *w* has been conserved during metazoan evolution as evident from the human and *Drosophila* cognate proteins sharing 34% identity and 58% similarity (J. Croop, personal communication). In *Drosophila*, *w* is required for pigment production in the light-screening cells of the compound eye, ocelli pigment cells, sheath cells of the testes, and the larval Malpighian tubules.

Communication between courting *Drosophila* involves an elaborate repertoire of gender-specific action patterns that transmit either acceptance or repelling signals (2, 16). Use of these signaling routines in a particular order and frequency is dependent upon dynamic feedback, with each partner modifying its behavior in response to signals received (17, 18). In *Drosophila melanogaster*, courtship between mature males is suppressed by their antiaphrodisiac pheromones and experience-dependent courtship modification (learned from the courting of immature males) as well as through the rejection signals males elicit in response to sexual advances (17, 19–21). Behavioral analysis of *Drosophila* mutants has revealed that genetic lesions that knock out single genes frequently have pleiotropic effects that impinge upon normal courtship behavior (reviewed in ref. 22). While the loss of white function does not alter the sexual preference of homozygous viable *w*<sup>−</sup> flies, absence of the light-screening pigments in its compound eyes impairs the male's ability to visually track a potential mate, thus reducing his "lights on" mating success when compared to

*w*<sup>+</sup> males (2, 3). In complete darkness, *w*<sup>+</sup> and *w*<sup>−</sup> males are equivalent in their mating efficiency (7). Female mating success is not diminished by the loss of *w*.

During the last decade, genetically engineered *w* constructs (mini-*w* genes), encoding full-length proteins, have been extensively used as eye markers in *Drosophila* to identify P-element-mediated germ-line transformants in *w*<sup>−</sup> recipient lines (5, 23, 24). Mini-*w* P-element vectors containing heat-inducible *hsp-70* promoter/transgenes (heat shock vectors) have been employed in numerous gain-of-function experiments studying the effects of ectopically expressed proteins *in vivo*. In this study, we show that (i) the heat shock vector's mini-*w* gene is also activated after heat induction, (ii) this misexpression of *w* leads to a marked change in the sexual behavior of mature adult males, and (iii) males who do not ectopically express *w* and normally repel homosexual advances will actively participate in homosexual courtship when exposed to a vigorous male–male courtship environment.

## MATERIALS AND METHODS

**P-Element Transformations and *Drosophila* Stocks.** All fly stocks were raised at room temperature (≈22°C) in plastic vials (Carolina Biological Supply, no. 17-3085) on instant fly food (Formula 4-24, Carolina Biological Supply, no. 17-3214) prepared according to the manufacture's specifications. Germ-line transformants were isolated using standard procedures (23) by coinjecting *Df(1)w67c2,y* embryos with the pHSBJCaSpeR vector (25) containing either a full-length *plx* cDNA in its sense ( $\alpha$  series) or antisense ( $\beta$  series) orientation relative to the vector's *hsp-70* promoter or just the vector and the helper plasmid p $\pi$ 25.7wc (see ref. 26; details of the *plx* cDNA isolation and its insertion in the vector are available upon request). Multiple, independent lines for each of the above constructs were established from *w*<sup>+</sup> G<sub>1</sub> adults. Chromosomal assignments of vector integrations were performed by out-crossing transformant males to *w*<sup>−</sup> females carrying dominantly marked second and third chromosomes. Chromosomal markers are described in Lindsley and Grell (27). Mini-*w* heat-shock transformant lines, established in other laboratories, that were tested for inducible homosexual courtship are the *hsp-sevA.ch21*, *hsp-sevA.ch41*, and *hsp-sevA.ch50* lines, all harboring transformation vector pW8 insertions with *hsp-70*/sevenless (*sev*) minigenes from the Hafen laboratory (28), the HS-STG3 (pWH1) and the RK-STG2 (pRK261) containing *hsp-70*/string (*stg*) genes established in the O'Farrell laboratory (29), and T8 and T9 containing *hsp-70*/murine HoxB9 transgenes within the pHSBJCaSpeR from the McGinnis laboratory (25).

**Mutagenesis and Genetic Crosses.** To knock out mini-*w* function, ethyl methanesulfonate (EMS) was fed to PHSBJ $\beta$ 2 males (second chromosome insertion) using standard procedures (30). Treated males were subsequently mated with females of the marked second chromosome balancer stock: *w/Dp(2;Y)A161,B<sup>+</sup>nub b Sco lt stw<sup>3</sup>/SM6a*. *w*<sup>−</sup> progeny were back-crossed to the balancer stock to establish four indepen-

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Abbreviation: EMS, ethyl methanesulfonate.

dent mini- $w^-$  stocks (from a screen of  $\approx 22,000$  flies). Two mini- $w^-$  lines were also established from this screen and served as positive controls. Crosses were set up using standard procedures with newly eclosed virgins. For unambiguous visual differentiation between PHSB $\beta_2$  transformants and *Df(1)w67c2,y* nontransformants, a PHSB $\beta_2$  stock that has a  $w^+y^+$  phenotype was established by crossing  $y^-$ ; PHSB $\beta_2$  males with females from the marked second chromosome balancer stock, which contains a  $y^+w^-$  X chromosome (described above). Resulting  $y^+w^+$  males were back-crossed to the balancer stock followed by sibcrosses between  $y^+w^+$  offspring to generate  $y^+$ ; PHSB $\beta_2$  homozygous flies. Males harboring PHSB $\beta_2$ CaSpeR insertions in a  $bw^-$  background were prepared by mating PHSB $\beta_3$  (third chromosome insertion) males to females with the following second and third chromosomes: *kis cn bw sp*/SM6a; *brm*<sup>20</sup> *l(3)mm*/TM6C. Their  $Cy^+$ , *Sb* sons (*kis cn bw sp*/+; PHSB $\beta_3$ /TM6C, *cu Sb e ca*) were crossed with *net bw sp* females and the resulting  $bw^-$ , *Sb*<sup>+</sup> sons were tested for chaining. To assess the effect of a  $st^-$  background, PHSB $\beta_1$  (X chromosome insertion) females were mated to  $w^-$  males with the following third chromosomes: *ru h th st cu sr e*<sup>5</sup> *Pr ca*/TM2. PHSB $\beta_1$ ; *ru h th st cu sr e*<sup>5</sup> *Pr ca*/+ sons were in turn crossed to females containing an attached X chromosome [*C(1)M4,y*] and the third chromosomes: *th st cp in ri Kg*<sup>9</sup> *Ki pP*/TM3. Males from this cross with a  $th^-$  and  $st^-$  phenotype were tested.

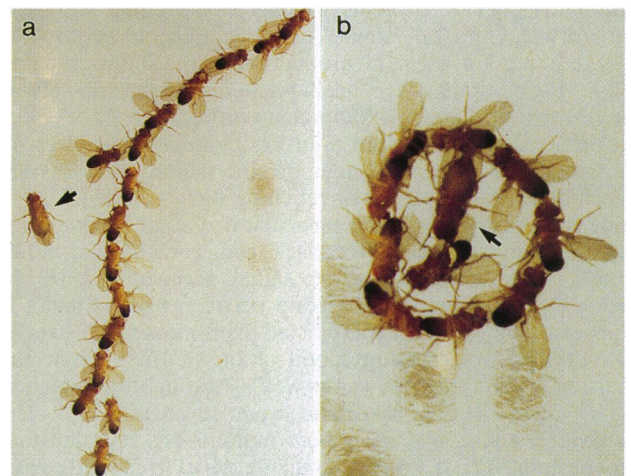
**Behavioral Analysis and Heat Shock Procedures.** Observation chambers were either wide-mouth 1-liter bottles (Nalge, no. 2107-0032) or 200-ml bottles (Corning, no. 25625-200), with screens on their tops (Spectrum Laboratories, Houston, no. 146464) and bottoms cut open to accommodate a standard 60-mm (diameter) Petri plate containing 20 ml of hardened grape juice/agar prepared according to Elgin and Miller (31). Unless otherwise stated, equivalent numbers of 3- to 10-day posteclosion adults (both sexes) were transferred into chambers containing yeasted grape juice/agar plates 18–20 hr prior to heat shock. For populations of 600–1500 flies, 1-liter bottles were used; the 200-ml observation bottles were used for observing <600 flies. One-hour, 37°C heat shocks occurred by transfer of the bottles to a lighted, humidified 37°C warm room. After heat shock, bottles were moved back to the room where its occupants were raised that was maintained under standard conditions of lighting (fluorescent), temperature ( $\approx 23^\circ\text{C}$ ), and humidity. Non-heat-shocked control populations (bottles containing equivalent numbers of adults taken from the same collection vials as the heat-shocked population) were not moved from this room. After shifting heat-shocked populations back to room temperature, they and their non-heat-shocked controls were observed for 5- to 10-min intervals every 0.5 hr for 4–6 hr. Populations were identified as possessing the male–male courtship trait if multiple, sustained courtship chains or circles of five or more were observed, none of which displayed courtship repelling signals (wing flicking, face kicking, and/or running away). Video recordings of the behavior were viewed in slow-motion to identify specific courtship action patterns (available upon request). Mixing experiments between  $y^+$ ; PHSB $\beta_2$  transformants and *Df(1)w67c2,y* nontransformants were carried out as follows: observation bottles with equivalent numbers of adults (4–5 days posteclosion) and differing ratios of transformants to nontransformants were prepared 18–20 hr prior to heat shock. Following heat shock, their behavior was observed as above over a period of 4–6 hr. Controls for each experiment consisted of bottles containing just transformants or nontransformants. Close examination of flies and their genitals was carried out with the aid of a dissecting microscope immediately following CO<sub>2</sub> anesthesia.

**RNA Detection.** Equal numbers of adult flies (both sexes, 3–10 days posteclosion) from each of the lines indicated in Fig. 2b were separated 20 hr prior to one-half receiving a 1-hr 37°C

heat shock. One hour after shifting the heat-treated populations back to room temperature, flies were anesthetized with CO<sub>2</sub> and immediately frozen in liquid N<sub>2</sub>. Northern analysis of poly(A)<sup>+</sup>-enriched RNA (8  $\mu\text{g}$  per lane), isolated from homogenized extracts, was carried out according to standard procedures (26). The *w* probe corresponds to a 2-kb *Sac* I fragment extending from the CaSpeR polycloning site to the *Sac* I site in its mini-*w* gene (32). The ribosomal protein 49 (rp49) probe was prepared according to O'Connell and Rosbash (33).

## RESULTS AND DISCUSSION

Initially, we noticed high levels of heat-inducible male–male courtship in transformant lines engineered to ectopically express *pollux* (*plx*) (ref. 31; unpublished data). Starting 0.5–1 hr after mature populations ( $\geq 3$  days posteclosion, both sexes) were shifted back to room temperature from a 1-hr 37°C heat shock, a marked change in the courtship behavior of the transformant male was observed. At this time, transformant males displayed their wings in a spread outward and upward position (Fig. 1; see Fig. 4). Close examination of these males revealed that many had protracted phalli. The en masse male wing posture and extended penises were not observed in heat-shocked control populations. By comparison, the sexual behavior of the transformant female was indistinguishable from those in control populations. Coincident with the extended-wings posture was the onset of vigorous male–male courtship. By 1 hr after heat shock (and continuing for 4–6 hr), male homosexual activity was predominantly observed in courtship chains (Figs. 1 and 2; see Fig. 4). Chain leaders frequently courted members of their own chains, creating courtship circles and lariats (Figs. 1b and 2; see Fig. 4). The male–male courtship activities included touching partners with forelegs, unilateral 90° wing extensions (a display that was followed by the extended-wings posture), licking the partner's genitalia, and curling the abdomen to achieve genital–genital contact (see Figs. 1 and 4). While participants repeated their courtship routines multiple times, no repelling signals were detected—i.e., wing flicking or face kicking (17). Courted males maintained an extended-wings posture while suitors licked their genitalia (see Figs. 1 and 4). Suitors rarely aban-



**FIG. 1.** Male homosexual courtship among PHSB $\beta_2$  adults. Male–male courtship activity was most apparent in courtship chains (a) and circles (b) in which participants repeated their courtship action patterns multiple times without eliciting repelling signals from their partners. Their courtship displays included touching partners with forelegs, unilateral wing extensions, genitalia licking, and curling of the abdomens to achieve genital–genital contact. When not displaying unilateral wing extensions, participants maintained their wings in an outward and upward position. Suitors rarely strayed from their partners to court nearby females (see arrows).

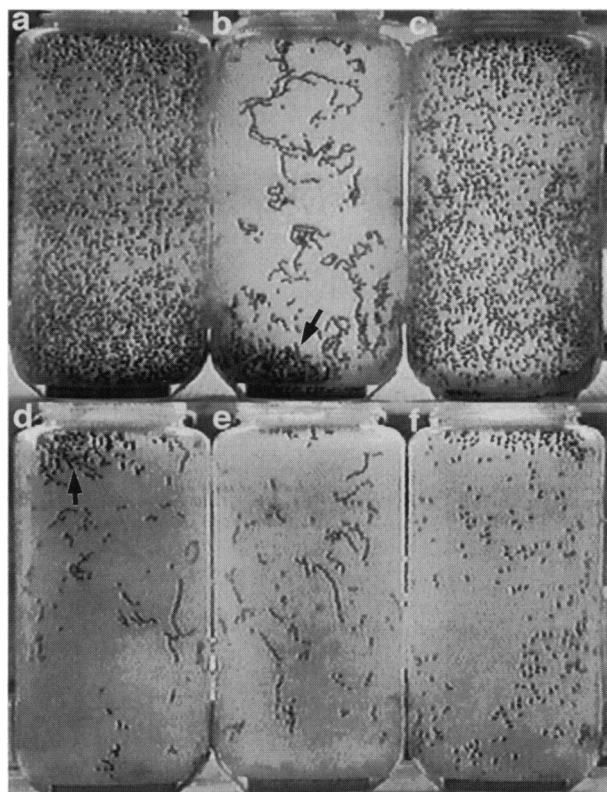


FIG. 2. Bottles a–c: Heat-inducible male homosexual behavior in transformant adults harboring heat-activated mini-*w* transgenes. Male homosexual courtship activity is evident from the silhouettes of the courtship chains, circles, and lariats. During vigorous courtship periods, most, if not all, males participated while females formed dense clusters (see arrows in the lower left side of bottle b and the upper left side of bottle d). Bottle a: Heat-shocked, nontransformant *Df(1)w67c2,y* flies. Bottle b: Heat-shocked PHSBJ $\beta_2$  transformants. Bottle c: Non-heat-shocked PHSBJ $\beta_2$  transformants. Bottles a–c each contain equivalent numbers of mature adults of both sexes ( $\approx 1500$  per bottle, 3–10 days posteclosion). Transformants in bottles b and c were raised in the same collection vials and separated 18 hr prior to heat shock. Bottles d–f contain equivalent numbers ( $\approx 700$  per bottle) of 3- to 10-day posteclosion PHSBJ $\beta_3$  adults. Bottle d: Both sexes. Bottle e: All males. Bottle f: All females. Note that transformant males do not require females to chain (bottle e). In the absence of males, transformant females did not form high-density clusters (bottle f). Views of bottles a–c and d–f are reproductions from video tape recordings 3.5 hr after heat shock.

done their partners to court nearby females (Fig. 1). During peak chaining periods (occurring 2–4 hr after heat shock), most, if not all, males participated.

Females occupied only lead positions in the courtship chains and in sexually mature populations (3 days posteclosion or older) their participation as chain leaders was brief. Lead males would typically break off their heterosexual advances after experiencing one or more of her repelling signals such as the extrusion of her ovipositor membrane (directed toward the advancing male's face), kicking his face, and/or running or flying away. During vigorous chaining periods, females predominately grouped together in tight clusters (see arrows in Fig. 2, bottles b and d); however, no female–female courtship was observed in or outside of these dense packs. In the absence of chaining males, heat-shocked transformant females did not form clusters but maintained a wide distribution throughout the bottle (Fig. 2, bottle f), indicating that female clustering was an avoidance response to vigorous male courtship activity. Female clusters were not observed in control bottles (Fig. 2, bottles a and c). The intense transformant male courtship activity also induced clustering in nontransformant females

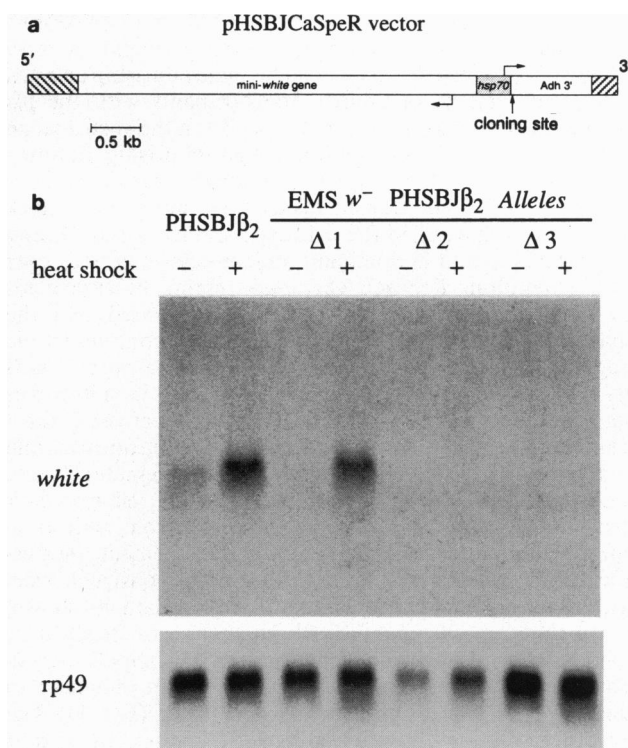


FIG. 3. Heat shock activates expression of the mini-*w* gene in PHSBJ $\beta_2$  transformants. (a) The pHSBJCaSpeR vector (25), derived from CaSpeR (32), contains 290 bp of the *hsp-70* promoter/transcription start site flanked by mini-*w* and the 3' untranslated trailer of the Alcohol dehydrogenase (*Adh*) gene (34). The reduced in size mini-*w* contains 300 bp of the endogenous *w* promoter, its transcribed sequence (except for most of its 5' intron), and 630 bp of DNA flanking its poly(A) addition site (32). Hatching represents P-element ends and the bent arrows indicate transcription initiation sites and directions for the mini-*w* and *hsp-70* promoters. (b) Northern analysis of *w* mRNA expression in PHSBJ $\beta_2$  adults and EMS-induced mini-*w*<sup>-</sup> alleles of PHSBJ $\beta_2$  with and without heat shock indicates that the *hsp-70* heat-responsive elements act as an enhancer to activate mini-*w* gene expression. To assess relative mRNA levels and integrity, the blot was reprobed with a ribosomal protein 49 (rp49) probe.

(data not shown). In mature populations, only inseminated females appeared to form these clusters; when sexually mature, virgin females were added to bottles containing chaining males, they copulated with males before joining the female clusters (data not shown). Female presence is not required for the male homosexual courtship activity; transformants can be induced to chain without females (Fig. 2, bottle e).

The inducible nature of the male–male courtship behavior is demonstrated by the lack of significant chaining in non-heat-shocked populations that were raised in the same collection vials but separated prior to induction (Fig. 2, bottles b and c). However, in the absence of heat shock, transformant males in populations that are 15 days posteclosion or older will form courtship chains during active periods (data not shown), indicating the *hsp-70* cis element may be activated by additional factors. If maintained on a 12-hr light/dark cycle, these events typically occurred shortly after lights on, at a time when males in nontransformant populations exhibited high levels of heterosexual courtship. Sexual maturity appears to be required for this activity: we have not detected chaining in newly enclosed (0–24 hr) transformants. Attempts to induce chaining in nontransformant populations by either transferring them into bottles that just had chaining males removed or by exchanging air between chaining and nonchaining populations have also failed.

Our *plx* cDNA was cloned into pHSBJCaSpeR (25, 32), a P-element vector containing the mini-*w* gene and the heat-

inducible *hsp-70* promoter (see Fig. 3a). All of our transformant lines, with insertions on either the X, second, or third chromosomes, showed inducible male–male courtship activity. Subsequent analysis of control transformants with the *plx* cDNA in its antisense orientation or with just the vector alone revealed the same behavior. Male–male courtship chaining was not detected in the recipient line for the transformations (Fig. 2, bottle a), in transformants containing heat shock vectors that lack *w*, or in the wild-type strains Oregon-R and Canton-S. This trait is dominant and successive crosses over three generations between vector-containing heterozygotes and their nontransformant siblings demonstrated that the behavior segregated with the chromosome containing the integrated vector. To determine if the parental line's *Df(1)w67c2,y* X chromosome is required or if its autosomes contained contributing recessive elements, we crossed transformant males (homozygous for vector insertions on either the second or third chromosomes) with Oregon-R females. Again, we observed the same inducible male–male courtship in their offspring. We have also observed this inducible activity in mini-*w*<sup>+</sup> containing heat-shock vector transformant lines established in other laboratories with different constructs, vectors, and/or parental lines (refs. 25, 28 and 29; data not shown; see *Materials and Methods*). Northern analysis of steady-state *w* mRNA levels revealed that the pHSBJCaSpeR *hsp-70* heat-activated cis element can function as an enhancer to activate mini-*w* expression in heat-shocked flies (Fig. 3b). The direction of mini-*w* transcription in our transformants and its general ectopic expression were verified by embryo *in situ* hybridization using strand-specific RNA probes (data not shown). To test the role of ectopic *w* function as the cause of this courtship behavior, we mutagenized one of our transformant lines with EMS and isolated four independent white-eyed alleles. None of the alleles displayed the inducible homosexual behavior; however, one of them continues to transcribe mini-*w* message (Fig. 3b). This message most likely encodes a non-functional protein: the expressing allele does not complement the *w*<sup>−</sup> X chromosome or the other EMS-induced mini-*w*<sup>−</sup> alleles. Taken together, we conclude that *w* misexpression has a profound effect on male sexual behavior. Following our personal communication of these findings, others, using transformant lines prepared outside our laboratory, have been successful in activating high levels of male–male courtship in mini-*w*<sup>+</sup> heat shock lines (A. Hing and J. Carlson, personal communication). However, one would expect that variable levels of homosexual activity may exist between different experiments and transformant lines, given that sexual behavior is most likely a manifestation of multiple integrated processes involving genetic and epigenetic factors.

To determine if transformant males would court nontransformants and vice versa, the two populations were mixed in different ratios. In a vigorous chaining environment ( $\geq 80\%$  transformants), we observed that nontransformant males (identified by their white eyes and yellow bodies) initially avoided homosexual encounters, but, with time, they began to actively participate (Fig. 4). At the beginning of the chaining period, transformant males actively courted both genotypes, while the behavior of the nontransformant was similar to that of males in control populations. The nontransformant male did not initially court other males, suggesting that the attraction transformants displayed toward other males was not the result of a misexpressed aphrodisiac pheromone. At this time, nontransformant males occupied only lead positions in rapidly moving chains and frequently displayed repelling signals in response to courtship advances (Fig. 4a). However, with increasing exposure to the vigorous courtship, the nontransformant males' involvement in homosexual activity increased significantly: by 2 hr after heat shock, they were frequently observed courting each other and occupying internal and trailing positions within chains (Fig. 4 b and c). Nontransfor-

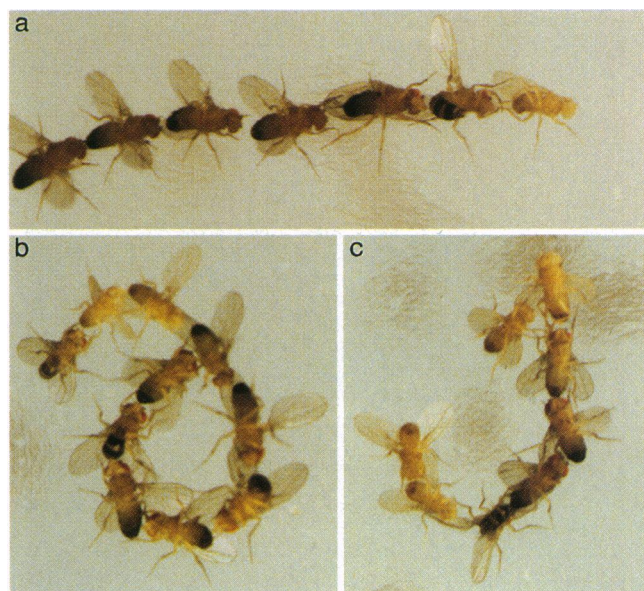


FIG. 4. Mixing experiments between transformants and nontransformants (identified by their yellow bodies and white eyes) revealed that nontransformant males actively participated in homosexual courtship when exposed to a vigorous chaining environment. (a) At the beginning of chaining periods, in bottles containing  $\geq 80\%$  transformants, nontransformant males were observed only at the head of fast moving chains where they repeatedly displayed repelling signals to advancing suitors—i.e., wing-flicking, face-kicking, and/or running away. (b and c) After a 2-hr exposure to vigorous transformant courtship, many, if not all, of the nontransformants were observed courting and inducing courtship.

mant chain participants did not display repelling signals and maintained extended wings in a manner similar to that of the transformant male. As the percentage of nontransformants to transformants increased, there was a corresponding reduction in chaining activity; in populations of  $\geq 50\%$  nontransformants, little or no chaining was detected and nontransformant participation was rarely observed. It remains to be determined which factor(s) present in a high-chaining environment persuades nontransformant males to join in. This participation may be the result of a natural response to a sexual cue such as the excitatory effect of the male courtship song. Males that are exposed to the courtship songs of other males are stimulated to court (35).

Two additional genes, scarlet (*st*) (36) and brown (*bw*) (37), are required for the uptake or distribution of tryptophan and guanine (respectively) in pigment-producing cells (14, 15). It has been proposed that White and Scarlet proteins form a heterodimer to transport tryptophan, while White and Brown interact to move guanine across membranes (36–38). Analysis of this behavior in *st*<sup>−</sup> or *bw*<sup>−</sup> mutants demonstrated that neither *st* nor *bw* functions are required for the inducible male–male chaining (data not shown), suggesting that White may interact with an additional factor(s) to trigger this behavior.

The apparent male-specific behavioral change brought on by *w* misexpression resembles the courtship behavior exhibited by the homozygous viable, autosomal recessive mutant fruitless (*fru*) (22, 39, 40). *fru* males vigorously court males, forming courtship chains with other *fru* males, while *fru* females display wild-type mating behavior. In addition, *fru* males have a reduced repelling wing-flicking behavior when compared to wild-type males and also display abnormal wing postures and courtship songs (40, 41). Although the courtship song of the mini-*w* transformant has not yet been analyzed, the behavioral similarities to the *fru* mutation suggest that the *fru* lesion and *w* misexpression may affect the same behavioral pathway(s).

The mechanism(s) by which *w* misexpression alters the sexual behavior of mature males is currently unknown. Ectopic expression of this tryptophan/guanine transporter may modulate levels of these raw materials in cells that utilize them for purposes other than pigment production and thereby affect additional physiological processes. For example, general ectopic expression may lead to reduced levels of the neurotransmitter serotonin by directly or indirectly lowering tryptophan (the serotonin precursor) in serotonin-producing neurons. Remarkably, depletion of tryptophan in rats and rabbits lowers serotonin levels and triggers male homosexual mounting behavior (42). Reduction of serotonin levels in cats also induces male homosexual activity (43, 44). Altering the level of guanine (the opterin precursor) may also impinge upon the metabolism of serotonin and dopamine by affecting the synthesis of tetrahydropterin. Tetrahydropterin is an essential cofactor in the synthesis of the serotonin and dopamine precursors 5-hydroxytryptophan and dihydroxyphenylalanine, respectively (reviewed in ref. 45). Taken together, these observations suggest that elements of the basic machinery controlling male sexual behavior may be highly conserved between taxonomically distinct organisms. Further genetic dissection of this inducible homosexual courtship may enhance our knowledge of the underlying mechanisms controlling sexual behavior.

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